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The overall goal of this project is to learn about the structure and assembly of a family of secretory polypeptides (SPs) which contain tandemly repeated sequences and have the ability to assemble into an insoluble fibrous polymer. During the past year, we accomplished the following. The gene for spl30 was identified and mapped to polytene chromosome region I-17-B. This gene produces a 3.6-kb mRNA whose expression is developmentally regulated. spl30 contains tandem repeats of a 14 amino acid sequence containing 29% Gly. A gene for a 6-kb poly(A) RNA was mapped to BR3 on chromosome IV. This gene, in contrast to the spl30 gene, is expressed constitutively. The protein product of the BR3 gene was tentatively identified as spl70 and contains an unusual periodic distribution of Cys residues. Solution turbidity was used to optimize conditions for assembly of SPs in vitro. Assembled SP-complexes were visualized in the electron microscope as a network of beaded fibers. Preliminary data from fractionation and reconstitution studies suggested that a mixture of SPs is required to achieve formation of the observed macromolecular complexes.

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SECRETORY POLYPEPTIDES ENCODED BY BALBIANI RING GENES

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Project Summary

The aim of this project is to learn about the structure, developmentally regulated synthesis and assembly of a family of secretory polypeptides (SPs) into an insoluble polymer of silk-like threads. SPs are exclusively synthesized in salivary glands of aquatic larvae of the Dipteran, Chironomus. All SPs studied to date are composed of tandemly repeated amino acid sequences. Recombinant cDNA probes are used to map SP-coding genes on polytene chromosomes, identify their mRNAs on Northern blots and derive the amino acid sequence of their encoded polypeptide. cDNA probes and anti-SP antibodies are used to study the level at which SP gene regulation occurs during normal larval development and under conditions of galactose-induced alterations in gene expression. SP assembly in vitro is being studied by a combination of physical, electron microscopic and biochemical methods. We hope to learn which SPs interact with each other and what is the chemical nature of these interactions. We eventually plan to determine the spatial distribution of SPs within assembled complexes by making three-dimensional tomographic reconstructions from immunoelectron micrographs. This experimental system provides a unique opportunity to study how naturally occurring soluble polypeptides can assemble into an insoluble fiber that functions in an aqueous environment. ~~_____~~

Results from the Prior Year

Identification and mapping of the sp130 gene. A cDNA clone was shown to hybridize to a 3.6-kb poly(A) salivary gland RNA. The hybridization intensity suggested that this transcript was abundant and/or contained internally repeated sequences. The nucleotide sequence of the 193-bp cDNA insert revealed that it contained similar 42-bp direct sequence repeats. An 18-nt synthetic oligonucleotide probe corresponding to a portion of one repeat was used to rescreen our randomly primed cDNA library. A total of five additional cDNA clones were isolated which exhibit various degrees of thermal stability with the oligonucleotide probe. These cDNAs are currently being sequenced and already exhibit the 42-bp repeat motif. Finally, in situ

hybridization of radioactive cDNA to polytene chromosomes demonstrated that the gene for this transcript was located at band 17B on chromosome I.

The mRNA-like strand of the cDNA insert was determined by differential hybridization to Northern blots and in all instances, two reading frames were open for protein translation. To identify the reading frame utilized in vivo and the polypeptide product of this putative mRNA, synthetic oligopeptides were synthesized. Affinity purified anti-peptide antibodies were used in Western blotting experiments to demonstrate that only one reading frame was used in vivo. The polypeptide product was a 130-kDal protein which was found to be secreted into the lumen of larval salivary glands; hence it was designated sp130.

The developmental expression of the sp130 gene was examined by a combination of run-on nuclear transcription assays (to determine the relative transcription rate), Northern blotting (to determine the steady-state level of sp130 mRNA) and Western blotting (to determine the glandular content of sp130). In summary, the sp130 gene exhibits a staircase pattern of developmentally regulated expression during the 4th larval instar. sp130 is almost undetectable between stages 1 through 4, a discrete level is detectable at stages 5 and 6, and the protein attains its highest concentration in salivary glands during the prepupal stages (stages 8 to 10). Transcription of the sp130 gene is maximal during prepupal stages giving rise to a steady-state level of between $2-6 \times 10^6$ mRNA molecules/secretory cell. Hence, the developmentally regulated expression of this gene seems to be primarily controlled at the level of transcription.

In summary, we have identified another SP-coding gene: the gene for sp130. Like other SPs, initial data suggest that sp130 will be largely composed of tandemly repeated amino acid sequences. However, the length (14 aa) and composition (29% Gly) of these repeats are distinct from other SPs studied to date. Furthermore, the localization of the sp130 gene to region I-17-B makes this the first SP-coding gene not found in a Balbiani Ring (BR). This distributes the SP-coding gene family over three chromosomes (I, III, and IV) and may have interesting implications for the evolution of the gene family. Finally, sp130 and sp180 are members of a special subset of SPs which are most abundant during the prepupal stages of larval development. We believe this is significant because the polymerized threads spun from prepupal salivary glands are structurally and microscopically different from the threads spun by younger larvae. This suggests that sp130 and/or sp180 may

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contribute directly to these observed differences. Future experiments will test this hypothesis directly.

Preliminary identification of a BR3 gene product. Another cDNA probe we are studying exhibited hybridization to Balbiani Ring 3 (BR3) on polytene chromosome IV. We are very excited about this finding because this is the only remaining uncharacterized BR. Northern blots indicated that the cDNA originated from a 6-kb poly(A)⁺ RNA. The cDNA insert contains over 480 bp but there are no direct sequence repeats. The mRNA-like strand contains only one open reading frame. The encoded polypeptide contains a periodic distribution of Cys residues: Cys₁-X-Cys₃-Y-Cys₅-...-Cys₁₂ occurs every 22 residues. We have a synthetic oligopeptide for one such unit and an affinity purified anti-peptide antibody. Preliminary Western blot data suggest that the polypeptide containing this sequence is sp170.

Developmental studies indicated that the relative transcription rate, steady-state level of mRNA and glandular content of sp170 remain essentially unchanged throughout the 4th larval instar. This is in contrast to our results for sp130 and sp180, but similar to our earlier findings for the 1000-kDal sp-I components. Together these results enhance the idea that polymerized secretory threads may be dynamic at least in part due to compositional changes in SPs.

In vitro assembly/disassembly of SPs. Considerable time and effort have been spent in developing an in vitro polymerization assay in which native SPs assemble into macromolecular structures. Assembled SP complexes scatter visible light; therefore solution turbidity measurements are made in a diode array spectrophotometer to measure the rate and extent of assembly. The assay buffer has been varied with respect to pH, ionic strength, various anion/cation combinations, the presence or absence of divalent metals and temperature. In all instances, the structural integrity of SPs was checked before and after assembly by SDS gel electrophoresis. Our current assembly buffer consists of 100 mM NaCl, 1 mM EDTA, 10 mM MES, pH 6.3. The complexes which assembled were reversible: solvent perturbation studies included the use of denaturants and reductants to learn about the types of interactions which stabilize these intermolecular structures. We concluded that the majority (80%) of solution turbidity which occurs during in vitro assembly of SPs can be abolished by denaturation. There was, however, a small percentage (20%) of turbidity which appeared to involve intra- or intermolecular covalent disulfide bonds. These conclusions are consistent with the primary structure of SPs predicted from the DNA sequence of their genes/mRNA.

Electron microscopy has been used as a complementary method to monitor assembly of SP complexes. This

enabled us to visualize morphological changes in macromolecular structures which may or may not be detectable as changes in turbidity. A variety of methods has been employed to maximize image contrast including negative and positive staining with uranyl acetate in aqueous and alcohol solutions with and without fixed angle or rotary shadowing with metal. Due to the variety in size, shape and complexity of the observed structures, no one method was suitable for all purposes. In general, maximum contrast of most types of images was obtained by allowing complexes to form on the surface of carbon-coated EM grids, positive staining with ethanolic uranyl acetate and fixed angle shadowing with platinum:palladium (80:20). SP complexes which exhibited appreciable solution turbidity appeared as a complex network of fibers periodically decorated with beads, particularly at fiber junctions. Fibers in the network often formed parallel arrays which sometimes coalesced into multistranded higher order structures approaching 400 nm in diameter. This is the diameter of the thinnest silk threads spun by larvae in vivo. Whereas reductants removed the beads from the fibers, the entire network was dissociated by a combination of reduction and denaturation. We concluded that the major component of the solution turbidity described above primarily originated from electrostatic interactions among SPs which form the fibrous network.

Several methods were employed to biochemically fractionate dissociated SPs. There were two objectives for these experiments. First, we wanted to examine fractionated SPs by electron microscopy to see if particular structures observed in assembled SP complexes could be ascribed to certain SP fractions. Secondly, we wanted to learn if any fractions or reconstituted mixtures of fractions could be reassembled in vitro. Velocity sedimentation in denaturing glycerol gradients was used to obtain three crude fractions of SPs. Gel electrophoresis indicates that: the fastest sedimenting fractions contained essentially pure 1000-kDal sp-I components; intermediate fractions contained a mixture of sp130, sp170 and sp150; the slowest fractions were mainly enriched in sp55 and sp60. Whereas the sp-I containing fractions appeared somewhat like lambda-shaped fibers, all other fractions appeared as an assortment of different size beads. This result implied that the sp-I components are the fibrous backbone of the networks observed in assembled SP-complexes and the beads may be any one of the other SPs. None of the SP-containing fractions assembled into macromolecular structures in vitro, however, a mixture of all fractions did appear to reconstitute structures which, in the electron microscope, appeared comparable to SP-complexes formed in vitro with unfractionated material. Therefore we concluded that the ability of SPs to form macromolecular complexes in vitro is due to the interaction of sp-I components PLUS one or more additional SPs.

Lastly, velocity sedimentation and gel electrophoresis have been used to ask if all SPs actually become part of the macromolecular complex that is assembled in vitro. When SPs were assembled in vitro and sedimented through nondenaturing glycerol gradients, a major complex sedimented at >1000 S. This complex contained all the SPs which were present in the starting material EXCEPT sp170. We concluded that either sp170 does not contribute structurally to the formation of the complex or that its association with the complex is weak.

Plans for Next Year

SP-coding genes. We will continue to identify and map additional SP-coding genes using recombinant DNA procedures which have been successful to date. It should be possible to confirm the identification of the BR3 gene product as sp170. One change in our selection criteria will be required: since the sp130 gene was mapped to chromosome region I-17-B, we will no longer disregard clones which do not hybridize to BRs. For example, the next cDNA to be analyzed this year is one which hybridizes in situ to region I-5/6 and originates from a 4.8-kb poly(A)⁺ RNA.

Our primary objective still remains to get a nucleic acid hybridization probe and antibody probe for each SP-coding gene and its polypeptide. To facilitate this, we have initiated a collaboration and exchange of probes with the labs of Professor J.-E. Edstrom and I.I. Kiknadze which have identified additional SP-coding genes in other species of Chironomus. In addition, we will, to a limited extent, begin to collect more sequence data for the overall structure of specific mRNAs. In particular we will begin by obtaining and comparing the sequence of the 3'-end of mRNAs for sp130, sp170 and sp180 between two or three species of Chironomus. Such interspecific comparisons were quite informative when the structure of (sp-I)-coding genes was deduced. This study will be made possible by the collaborative efforts of the labs mentioned above.

Developmental studies of SP gene expression. All newly discovered SP-coding genes will be examined for developmental patterns of expression to learn when particular proteins are synthesized. In addition, we will reexamine galactose-induced alterations in SP gene expression. Earlier studies were done before we knew about the developmental expression of sp130 and sp180 genes and the gels were run under conditions which only resolved sp-I components. We plan to find out whether early and late 4th instar larvae respond similarly to galactose with respect to which SP-coding genes are induced, repressed or unaltered in their expression.

Structural organization of SP complexes. We will continue to use glycerol gradient centrifugation to study SP-SP interactions. By sedimenting complexes exposed to increasing concentrations of denaturing/reducing agents, we hope to establish a hierarchy of SP-SP associations. We will also investigate whether or not the presence of sp170 is required for macromolecular complex formation. We will continue to pursue further fractionation of SPs with the hope that once we identify the minimum constituents required to form a complex, "mixing and matching" experiments may enable us to test our hypothesis that the addition of prepupal SPs (sp130 and sp180) may contribute to the formation of silk threads with altered structures and physical properties.

Finally we hope to initiate the microscopic identification of SPs in complexes assembled in vitro and in vivo. To begin with, anti-peptide antibodies will be used on partially fractionated SPs and unfractionated complexes to see if we can localize and identify specific polypeptides. These will be pilot studies for the following year when we try to simultaneously identify two or more SPs within a complex. This will require antibodies raised in different animals, hence this year we will raise mouse monoclonal anti-SP antibodies. These antibodies will be indispensable reagents for our future immunoelectron microscopic studies, particularly those involving electron microscope tomography.

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